

THE PHYSIOLOGY OF INSECT DIAPAUSE. VII. THE RESPIRATORY METABOLISM OF THE CECROPIA SILKWORM DURING DIAPAUSE AND DEVELOPMENT¹

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The endocrine control of the pupal diapause of the Cecropia silkworm has been the principal theme of the preceding papers of this series. A considerable body of evidence is now at hand pointing to the prothoracic glands as the source of a hormone which reacts with the tissues to terminate the pupal diapause. The prothoracic gland hormone, in turn, has been identified as the "moulting hormone," the "pupation hormone," and the "growth and differentiation hormone" of previous investigators (*cf.* Williams, 1952).

The present paper initiates a series of reports in which the analysis is narrowed to the reaction of the hormone with the pupal tissues. The central though necessarily distant objective is to define the biochemical events which couple the action of the hormone to the biological end-results. It seemed possible that this elusive goal might be particularly accessible in the case of the diapausing insect where the prothoracic gland hormone produces prompt and spectacular effects in each and every tissue. As a first step in this direction the present study considers the respiratory metabolism of the Cecropia silkworm during diapause and development.

MATERIALS AND METHODS

1. *Experimental animals*

The present investigation is based on approximately 1500 respiratory measurements performed on a total of 150 larvae, pupae, and developing adults of the giant silkworm *Platysamia cecropia*. One series of experiments was carried out on pupae of the closely related *Cynthia* silkworm (*Samia walkeri*). The insects were reared from eggs and managed according to techniques described previously (Williams, 1946).

2. *Apparatus*

Most of the measurements were performed at 25° C. in 45-cc. vessels equipped with venting-plugs and adapters for use with standard Warburg manometers (Fig. 1). In occasional experiments the gas volume of the system was decreased by the insertion of lucite "space-occupiers" into one or more vessels. Certain preliminary experiments were carried out in 25-cc. non-compensated capillary respirometers.

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FIGURE 1. Respirometer vessel for use with standard Warburg manometer. The vessel is equipped with a side-arm, a venting-plug, and an adapter for junction with the manometer. The pupa is supported on a glass tube above alkali-moistened filter paper. The lucite cylinder on the extreme right is a space occupier for increasing the sensitivity of the system.

The carbon dioxide output was absorbed on a strip of filter paper moistened with 0.3 to 0.5 cc. of 1.5 N sodium hydroxide. Measurements were performed over periods that averaged twenty hours, the excursions of the manometer being recorded at intervals of from five minutes to eight hours, as dictated by the rate of oxygen uptake.

At the conclusion of each experiment, acid was added to displace the carbon dioxide from the alkali, and the volume of the evolved gas determined in an analyzer of the type described by Bliss (1953). The several precautions outlined by Scholander *et al.* (1952) for experiments of this type were observed. The total output of carbon dioxide was determined from the moment the vessels were sealed until the end of the experiment, and the average calculated over this period. The

over-all respiratory quotient of each insect was calculated by dividing the average rate of carbon dioxide output by the average rate of oxygen consumption.

OXYGEN CONSUMPTION DURING METAMORPHOSIS

Enormous changes were observed in the rate of oxygen consumption of the *Cecropia* silkworm during the course of its metamorphosis at 25° C. In Figure 2 these changes have been assembled and plotted, the discontinuity in the x-axis corresponding to the several months of temporary storage at 5° C. prior to the

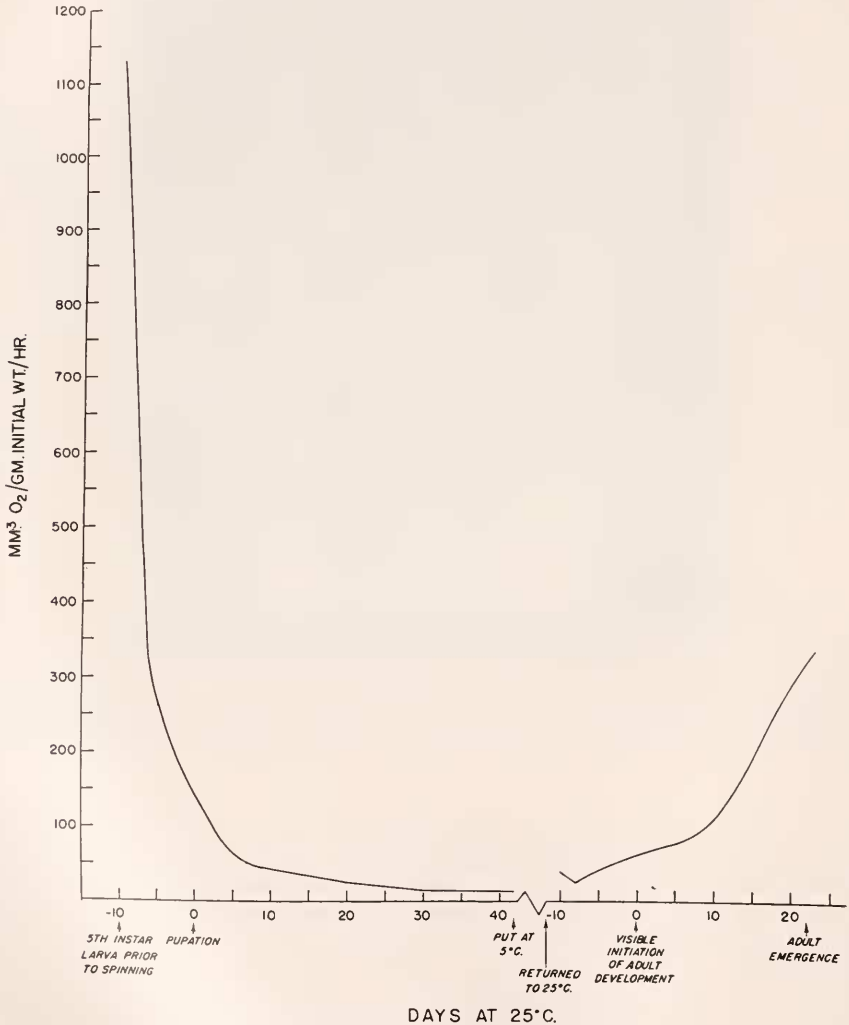


FIGURE 2. The average rate of oxygen consumption at 25° C. of the *Cecropia* silkworm during its life history. The rates of oxygen consumption are expressed as mm^3 . oxygen per gram initial weight per hour; the "initial weight" refers to the weight of each individual two to three weeks after pupation.

return of the diapausing pupae to 25° C. In each instance the rates of oxygen consumption of the various animals have been computed in terms of the live weight of each individual several weeks after pupation. This procedure, the use of the pupal weights in the computations, automatically compensated for the large weight loss of the mature larva prior to pupation and of the pupa during the course of adult development.

The several segments of the curve will now be considered in further detail.

1. Larva, prepupa, and pupa

Figure 3 illustrates the precipitous decrease in the rate of oxygen consumption of *Cecropia* during the larval-pupal transformation. Similar measurements are recorded in Figure 4 for the closely related *Cynthia* silkworm. The animals were maintained at 25° C. and all measurements performed at this temperature.

It is clear that a striking decrease in the insect's metabolism begins just after the cocoon has been spun and continues throughout the prepupal and early pupal periods. By the second week after pupation the rate of oxygen consumption, in the case of *Cecropia*, is 2.5 per cent of that of the mature larva. By about the third week following pupation the rate of oxygen consumption reaches an extremely low level where it persists during the many months of pupal diapause at 25° C.

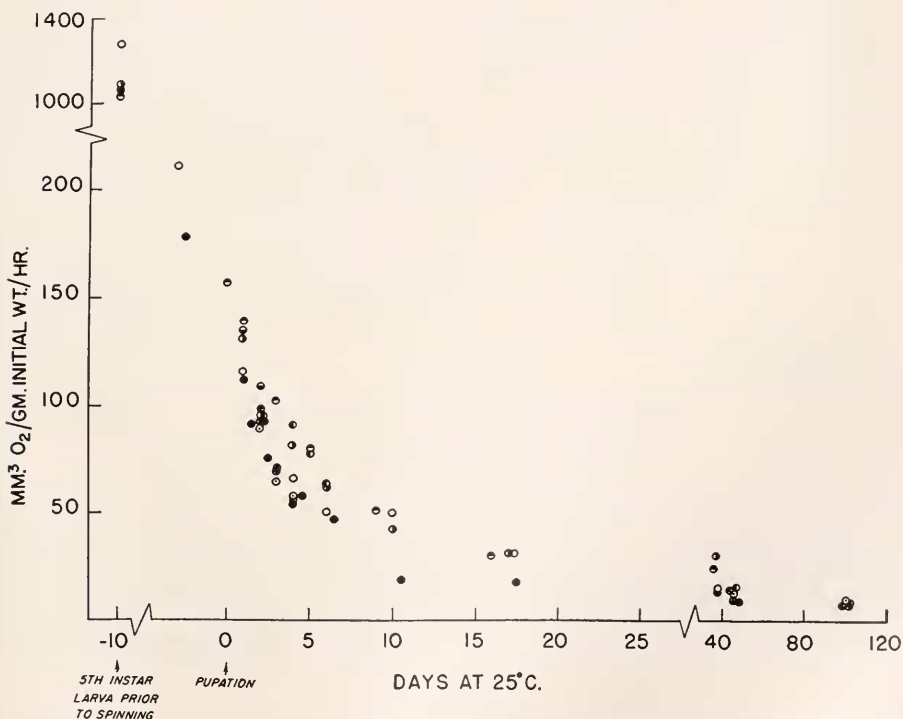
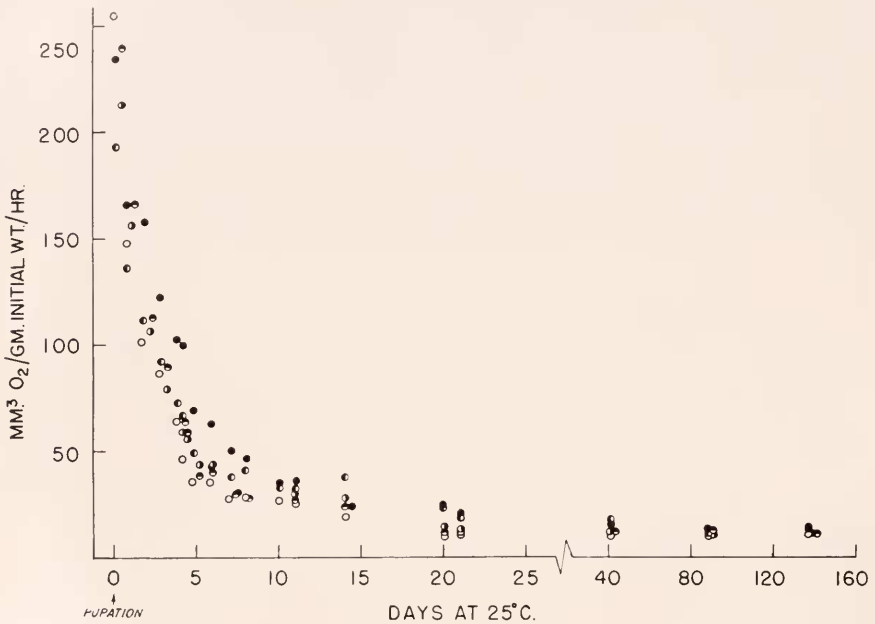


FIGURE 3. The rate of oxygen consumption of mature fifth instar *Cecropia* larvae at 25° C. just prior to spinning (ten days before pupation) and at frequent intervals thereafter. The measurements on larvae are determinations during periods when the animals were quiescent within the respirometers.

Ten to sixteen weeks after pupation, 43 diapausing *Cecropia* pupae, varying in weight from 4 to 6 gm., showed an average oxygen consumption of 16.3 ± 9.03 (s.d.) mm^3 per gram live weight per hour. The median was 13.7 and the extremes 6.8 to 38.0. On a dry weight basis the average was 65.0 mm^3 per gram per hour. These values are but 1.4 per cent of the corresponding measurements of mature larvae and 11 per cent of those of fresh pupae.

2. Previously chilled pupae prior to the initiation of adult development

Figure 5 records the respiration of five previously chilled pupae at frequent intervals after being returned to 25°C . The first visible signs of adult development



at 25° C. The results are typical of a number of experiments of this type. The initiation of adult development is characterized by a continuation of the increasing metabolism which, as we have just seen, had already been evident a week before the visible initiation of development. During the final two-thirds of adult develop-

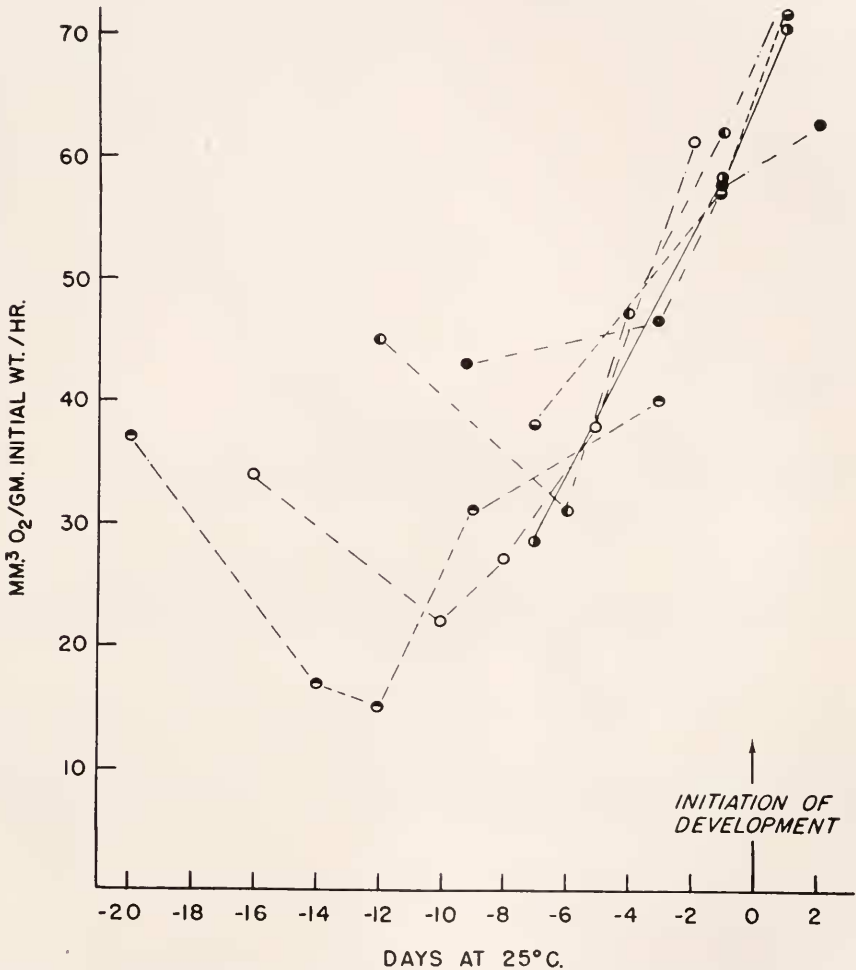


FIGURE 5. Rates of oxygen consumption of five previously chilled pupae after return to 25° C. The initial measurements for each animal were made two days after removal from the low temperature. The respiratory metabolism begins to increase about one week prior to the visible initiation of adult development.

ment, the rate of oxygen consumption increases yet more rapidly. Just prior to emergence, the average respiration of five fully formed adult moths was 312 mm³ of oxygen per gram initial live pupal weight per hour. This value is 19 times that of diapausing pupae, but only $\frac{1}{3}$ that of mature larvae.

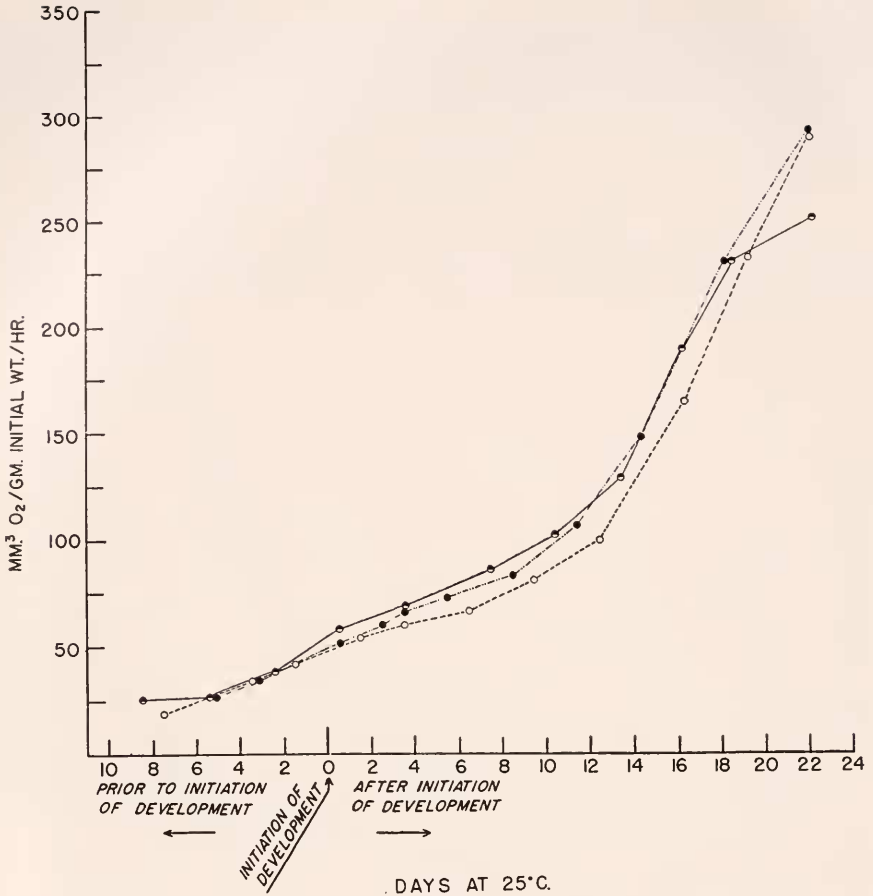


FIGURE 6. Rates of oxygen consumption during the course of the adult development of three previously chilled pupae. In this group of animals the adults emerged 24 days (average) after the initiation of adult development.

THE RESPIRATORY QUOTIENT DURING DIAPAUSE

1. Discontinuous release of carbon dioxide by diapausing pupae

The respiratory quotients of diapausing pupae were studied at 25° C. in animals previously stored for several months at this same temperature. In preliminary experiments an astonishing degree of scatter was encountered in the respiratory quotients when the latter were determined over one-hour intervals. Individual animals showed quotients which varied from 0.1 to 3.0 during successive hours—a result which suggested that either the intake of oxygen and/or the release of carbon dioxide were discontinuous.

Though a detailed study of the matter will be considered in a subsequent communication, we can state that experiments testing these possibilities have provided results of the type illustrated in Figure 7. Whereas the rate of oxygen utilization

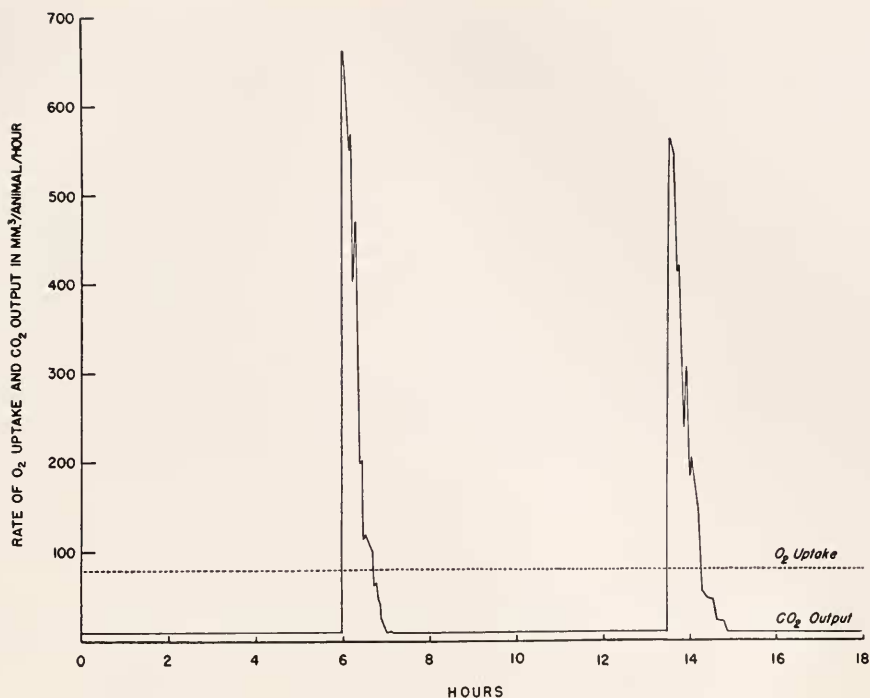


FIGURE 7. Rates of oxygen consumption and carbon dioxide output of a diapausing *Cecropia* pupa at 25° C. over an eighteen-hour interval. The "direct method" of Warburg was employed in this experiment.

was essentially constant, an extraordinary degree of discontinuity was observed in the rate of output of carbon dioxide. The latter was "blown off" in "bursts" every few hours, less than 10 per cent being excreted continuously.

We have consistently observed this phenomenon in all diapausing lepidopterous pupae examined—a finding in substantial agreement with the detailed results reported by Punt (1948, 1950). For our present purposes the discontinuous release of carbon dioxide is of interest because it illustrates a serious problem in the estimation of the respiratory quotients of diapausing pupae. It is clear that only prolonged measurements can provide a reasonable indication of the ratio between carbon dioxide release and oxygen consumption.

2. Respiratory quotient of diapausing pupae

The respiratory quotients of 49 diapausing *Cecropia* pupae were determined at 25° C. after three to five months of storage at this same temperature. Each experiment was continued for at least 17 hours in order to encompass two or more carbon dioxide bursts. The average respiratory quotient was 0.78 ± 0.093 (s.d.); the medium was 0.77 with extremes of 0.57 to 1.00. A similar series of determinations on seven brainless diapausing pupae recorded an average respiratory

quotient of 0.72 ± 0.091 (s.d.); the median was 0.72 with extremes of 0.57 to 0.87. Consequently, there appears to be nothing peculiar about the average respiratory quotient of diapausing *Cecropia* pupae, provided that the experimental technique is adequate to cope with the discontinuous release of carbon dioxide.

STIMULATION OF PUPAL METABOLISM BY LOCALIZED INJURY

In experiments testing the metabolic effects of various drugs when injected into diapausing *Cecropia* pupae, a prompt increase in the rate of oxygen consumption was consistently observed. It soon became evident that the effect was due, not to the injected drug, but to the perforation of the integument in the course of the injection. For example, the same result was induced by puncturing the integument with a dry needle. The phenomenon was studied in further detail.

The effects of minor injury to the integument are illustrated in the data summarized in Table I. In this experiment the average rates of oxygen consumption and carbon dioxide output were first measured at 25° C. in a series of nine dia-

TABLE I

Effects of small integumentary incisions on the respiration of diapausing Cecropia pupae at 25° C. The anti-tyrosinase, phenylthiourea, was implanted into three of the nine individuals at the time of incision (Group B)

Experimental time	Group A No PTU when incised (6 animals)		Group B Received PTU when incised (3 animals)		Over-all average (9 animals)	
	Relative rate of oxygen consumption	R. Q.	Relative rate of oxygen consumption	R. Q.	Relative rate of oxygen consumption	R. Q.
1 day prior to incision	100%	.81	100%	.88	100%	.84 ± .13 (s.d.)
1 day after incision	272%	.81	313%	.81	287 ± 59 (s.d.) %	.81 ± .06 (s.d.)
6 days after incision	183%	.84	193%	.72	186 ± 42 (s.d.) %	.80 ± .09 (s.d.)
22 days after incision	77%	—	81%	—	78 ± 16 (s.d.) %	—

pausing *Cecropia* pupae. A V-shaped incision having a total length of 4 mm. was then made in the thoracic tergum of each individual just lateral to the mid-line. Into three of the animals crystals of the anti-tyrosinase, phenylthiourea, were implanted at this time. The sites of injury were then sealed with melted paraffin. Twenty-four hour measurements of the rates of oxygen consumption and carbon dioxide output were then performed every few days.

The rate of oxygen consumption of each individual began to increase within one hour and became maximal within twenty-four hours; at this time it averaged 287 ± 59 (s.d.) per cent of the pre-injury values (extremes of 224 and 424 per cent). Six days after the injury the rates of oxygen consumption were still elevated at 186 ± 42 (s.d.) per cent of the initial values. By the twenty-second day the injury metabolism had totally disappeared and the rate of oxygen consumption had returned to 78 ± 16 (s.d.) per cent of its value prior to injury.

Table I also indicates that the potent anti-tyrosinase, phenylthiourea, was with-

out effect in opposing or preventing the injury metabolism. Moreover, since the respiratory quotient remained unchanged after injury, it is clear that the oxygen consumption and carbon dioxide production were stimulated in the same proportion.

The preceding experiments were performed on pupae capable of moving their abdominal segments. It seemed possible that the injury metabolism had its source in increased activity of the abdominal musculature. Two lines of experimentation were performed to test this possibility. In the first of these, the effects of injury were studied on isolated anterior and posterior ends of diapausing pupae. The injury metabolism was found to be just as pronounced in the immotile anterior fragments as in the motile posterior fragments. In the second type of experiment, a series of four pupae was immobilized by the removal of the entire chain of abdominal ganglia and connectives, thereby denervating the intersegmental muscles of the abdomen. Two months later the rates of oxygen consumption were measured, following which the thoracic tergum of each individual was punctured with a dry 22-gauge hypodermic needle inserted to a depth of 4 mm. Two hours after the injury the rates of oxygen consumption showed an average increase of 42 ± 17.5 (s.d.) per cent. It is clear that muscular activity is not responsible for the injury metabolism.

TABLE II

Effects of extensive injury to the integument on the respiration of nine diapausing Cecropia pupae at 25° C.*

Experimental time	Relative rate of oxygen consumption		
	Average	Median	Range
1 day prior to operation	100%	96%	69-145%
3 days after operation	814%	750%	570-1450%
69 days after operation	291%	207%	101-716%

* The integument overlying the pupal legs was removed and replaced by a triangular plastic slip sealed in place with melted paraffin. Crystals of phenylthiourea were implanted into each animal at the time of injury.

The magnitude and persistence of the injury metabolism appeared to vary with the extent of the injury. Major surgical procedures caused a tremendous increase in the rate of oxygen consumption which persisted for long periods thereafter. This fact is illustrated in the experiments summarized in Table II.

It is of particular interest and importance that diapausing pupae showed no over-all morphological change in response to the injury metabolism. Even when the metabolism was increased as high as 14-fold, the pupae continued to diapause—a fact confirmed by day-to-day observations and by dissections at the conclusion of the experiments.

DISCUSSION

Just prior to the spinning of the cocoon the mature fifth instar *Cecropia* larva ceases the intake of substances other than atmospheric oxygen. Henceforth until its death as a mature moth ten months later, the insect reworks according to its needs the materials of its own body. The larva, whose metabolism per unit mass

ranks among the highest recorded for non-flying invertebrates, transforms into a diapausing pupa, whose metabolism is among the lowest noted for any animal (*cf.* Robie, 1949; Heilbrunn, 1952). Then, months later, yet another transformation converts the diapausing pupa into the adult moth—a flying machine whose metabolism during flight is approximately two thousand times that of the pupa.

These results (Fig. 2) are in substantial agreement with those described by Heller (1926) for the diapausing sphingid, *Deilephila*. Like Heller, we observe that the months of pupal diapause are characterized by an extremely low metabolic rate, corresponding to the horizontal portion of the U-shaped curve of metabolism.

It is not our present purpose to consider the significance of the U-shaped curve in detail, but only to call attention to the metabolic characteristics of the two great periods of rapid rearrangement and maximal morphogenetic change. During the first of these, the prepupal period, the respiratory metabolism *decreases* more precipitously than at any other stage in the life history. During the second period of rapid change, the first few days of adult development, the over-all respiratory metabolism is extremely low—lower, in fact, than that which one can induce by injury to pupae during diapause. It is indeed true, as indicated in Figure 2, that the overall metabolism undergoes considerable increase during the latter stages of adult development. But this change mirrors the construction and maintenance of the future flying machine and, in our opinion, has no direct bearing on the earlier events which terminate the pupal diapause.

For these several reasons we are unimpressed by arguments which emphasize the high metabolic cost of morphogenesis and attribute the state of diapause to a simple quantitative deficiency in the over-all respiratory metabolism.

All of our experiments indicate that the oxygen consumption begins gradually to increase about one week prior to the visible initiation of adult development. Boell (1935) records a similar observation on diapausing grasshopper embryos where changes in respiratory rate were detected two days before the development of the embryo became apparent. This increment of respiration apparently signals a change in the pre-existing steady state of diapause and, in the case of *Cecropia*, suggests that the biochemical processes responsible for the initiation of adult development are set in motion about a week before one can detect any morphological evidence of the termination of diapause. The visible initiation of development is evidently the end-product of a brief period of endocrinological and biochemical preparation. Since the onset of adult development is brought to pass by the reaction between the growth and differentiation hormone and the diapausing tissues, the respiratory measurements suggest that the secretion of this hormone at 25° C. begins about a week before the actual initiation of adult development.

Turning to a consideration of the respiratory quotient, the measurements on diapausing *Cecropia* reveal nothing unusual, aside from the fact that the carbon dioxide is given off intermittently. A total of 49 determinations based on prolonged measurements showed an average respiratory quotient of 0.78 ± 0.093 (s.d.)—a value consistent with the combustion of a mixture of fat, protein, and carbohydrate. Though the respiratory quotients of diapausing lepidopterous pupae have hitherto been studied only by Agrell (1947, 1951a), the above-mentioned value is in good agreement with numerous determinations previously reported for non-diapausing pupae and for the changes occurring in the corresponding substrates (for summary see Needham, 1942, and Portier, 1949).

Our findings on *Cecropia* therefore stand in marked contrast to those described by Agrell (1947, 1951a) for the diapausing lepidopterous pupae, *Phalera* and *Endromis*. In measurements performed at 18° C. Agrell recorded respiratory quotients ranging from 0.1 to 0.3. On the basis of these extremely low values, Agrell has proposed a comprehensive theory of diapause (1951a, 1951b). According to this theory, the enzymes serving decarboxylation are defective in diapausing pupae due to a deficiency in thiamine and other vitamins acting as prosthetic groups in decarboxylases. The hormone which terminates diapause does so, he suggests, by promoting the synthesis of these particular vitamins, thereby reconstituting the decarboxylases. Previously thereto, the low respiratory quotients of diapausing pupae signal large scale conversion of fats into carbohydrates.

The respiratory quotients which formed the factual basis of this theory were determined by Agrell using the Thunberg microrespirometer. Each respiratory quotient was based on measurements over three to five successive periods of about one hour each. Agrell reports that the respiratory quotients obtained during these successive periods were uniform—a result indicative of a steady consumption of oxygen and release of carbon dioxide. But in our experience, as well as in that of Punt (1948, 1950), the carbon dioxide output of all the diapausing lepidopterous pupae examined (Sphingidae, Saturniidae, Noctuidae, Papilionidae) is characterized by singular discontinuity. In *Cecropia* at 25° C. less than 10 per cent of the carbon dioxide is released continuously. The remaining 90 + per cent is stored for periods averaging eight hours and then released within a one hour period (unpublished observations).

Though Agrell (1951a) suggests that the low respiratory quotients which he reports had (p. 283) “nothing to do with the rhythmic expulsion of carbon dioxide,” it is difficult to be certain of this fact. Unless *Phalera* and *Endromis* differ from other genera and species of diapausing Lepidoptera, the release of carbon dioxide occurs during short, widely spaced intervals rarely encompassed within brief periods of measurement. In these same terms the increase in the respiratory quotient which Agrell observed at the outset of adult development could be accounted for, since the release of carbon dioxide is known to be less discontinuous after adult development has been initiated (Punt, 1948, 1950) and a representative sample could probably be obtained during brief measurements.

Since the determinations of *Cecropia* were performed over periods of not less than 17 hours, we conclude that the average respiratory quotient of 0.78 is a valid indication of the type of metabolism of diapausing *Cecropia* pupae, and that it probably signifies the combustion of a mixture of fats, proteins, and carbohydrates. Agrell's theory of diapause therefore appears to be unacceptable in the case of the *Cecropia* silkworm.

The injury-stimulated metabolism of *Cecropia* pupae is a phenomenon no less curious than the discontinuous release of carbon dioxide. A simple perforation of the integument reacts on the animal as a whole to cause a prompt and pronounced increase in the rates of oxygen consumption and carbon dioxide production. In the case of more extensive injury, the metabolism increases as much as 14-fold and requires longer than 2½ months to return to normal.

These results have certain parallels in the literature. Agrell (1951a) reports

that the injection of physiological saline caused an 80 per cent increase in the respiration of diapausing pupae of *Endromis*, while Sussman (1952) has previously noted the phenomenon in the case of *Cecropia*. In *Calliphora* adults, injury is without effects on oxygen consumption (Thomsen, 1949); in *Periplaneta* adults, the injury-stimulated respiration persists for only 5 hours (Hassett, 1948); in the unfertilized eggs of *Bombyx*, only 24 hours (Wolsky, 1939). In this connection the large and prolonged changes in the colligative properties of the blood which Robinson (1928) observed after integumentary injury to diapausing pupae of the giant silkworm, *Telea polyphemus*, are of special interest.

The injury metabolism, as we have seen, is characterized by a proportional increase in both oxygen consumption and carbon dioxide output. The effect is not a by-product of increased muscular activity, since it persists in fragments devoid of somatic musculature and in animals in which the muscles have been denervated. Finally, the increased metabolism is insensitive to phenylthiourea and, consequently, is not mediated by tyrosinase.

Though the basis of the injury-stimulated respiration remains undefined, it is clear that the metabolism which it mirrors cannot be equated to the metabolism of the developing post-diapausing insect. For, though the rate of respiration of an injured diapausing pupa can increase to a level characteristic of animals halfway through adult development, no development occurs. Thus, the diapausing pupa, while capable of respiring at rates that characterize the growing insect, fails to grow in the absence of the proper hormonal stimulus and the proper *quality* of metabolism. It would appear that the increment of respiration induced by injury cannot be coupled to morphogenesis.

The injury-stimulated respiration, in itself, gives assurance that the absence of morphogenesis during diapause is not attributable to a simple *quantitative* deficiency in the dehydrogenase enzymes which release hydrogen from substrate, or in the redox enzymes which transmit the hydrogen to oxygen.

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SUMMARY

1. The over-all metabolism of the *Cecropia* silkworm, as signaled by its respiratory exchange, undergoes pronounced alterations during the course of the larval-pupal-adult transformation.

2. The rate of oxygen consumption of the diapausing pupa, averaging at 25° C. 16.3 ± 9.03 (s.d.) mm.³ per gram live weight per hour, is only 1.4 per cent that of the mature larva and 5 per cent that of the adult moth just prior to ecdysis. The metabolism at 25° C. persists at or near this low level throughout the pupal diapause.

3. One week prior to the termination of diapause the metabolism begins gradually to increase. This change apparently accompanies the initiation of the endocrine events which culminate in the termination of the pupal diapause. By the first day of visible adult development the rate of oxygen consumption is $3\frac{1}{2}$ times that of the diapausing pupa.

4. The continuation of adult development after the termination of diapause is accompanied by further increase in the rate of respiratory exchange—a change which mirrors the differentiation of the adult moth.

5. It was found that localized perforations or other injuries to the integument of diapausing pupae caused a pronounced increase in the insect's metabolism. The magnitude and persistence of the enhanced respiration varied with the extent of the initial injury. After extensive injury, the metabolism increased up to 14-fold and required over 2½ months to return to initial levels. The injury metabolism was unaffected by the anti-tyrosinase, phenylthiourea.

6. Though the metabolism of diapausing pupa could in this manner be increased to levels characteristic of the post-diapausing insect mid-way in adult development, no development took place. The significance of this finding is considered in relation to the biochemical basis of diapause.

7. It was found that whereas the utilization of oxygen by diapausing pupae is continuous, the release of carbon dioxide is discontinuous. Ninety per cent of the carbon dioxide is accumulated and "blown off" in short "bursts" which, at 25° C., occur about once every eight hours. This fact necessitated prolonged experiments for the determination of respiratory quotients.

8. In experiments averaging 20 hours in duration, 49 diapausing *Cecropia* pupae showed an average respiratory quotient of 0.78 ± 0.093 (s.d)—a value consistent with the combustion of a mixture of fats, carbohydrates, and proteins during diapause.

9. No indication was found of the extremely low average respiratory quotients, the multiple deficiencies in vitamins serving decarboxylation, or the conversion metabolism which Agrell has postulated as the biochemical basis of the pupal diapause.

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